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(54) Metalloproteinase inhibitor.

(57) A novel metalloproteinase inhibitor, analogs thereof, polynucleotides encoding the same, and methods of production, are disclosed. Pharmaceutical compositions and methods of treating disorders caused by excessive amounts of metalloproteinase are also disclosed.

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Murray et al. [J. Biol. Chem. 261, 4154-4159 (1986)] reported the purification and partial amino acid sequence of a bovine cartilage-derived collagenase inhibitor. The amino-terminal amino acid sequence of bovine MI of the subject invention is very similar to that reported by Murray et al. for the bovine cartilage-derived collagenase inhibitor (94% homology over first 38 residues), and the amino acid compositions are similar also. Murray et al. (J. Biol. Chem., supra) pointed out that the bovine cartilage-derived inhibitor had greater than 65% homology to human TIMP over the first 23 residues and that the amino-terminal sequences were "quite similar." Until the present work, no additional molecules related to or homologous to TIMP had ever been isolated from the same species from which a TIMP had been isolated. In the present work, two metalloproteinase inhibitors have been isolated and purified, and extensively characterized, from the same species (bovine) and indeed from the same cell conditioned medium. It is therefore clear that although they are related, as indicated, they cannot both be the bovine homolog of TIMP. One of them (peak II-derived), also as indicated, is probably bovine TIMP. The other (peak I-derived) must consequently be a new and additional molecule. Based on this discovery, it is apparent, for the first time, that there is a homologous inhibitor additional to TIMP encoded by the human genome. This human gene, i.e., the human MI gene, is set forth in Example 3.

To the extent that metalloproteinase inhibitors such as those described herein may prove to be therapeutically significant and hence need to be available in commercial scale quantities, isolation from cultures of naturally-occurring cells is unlikely to provide an adequate source of materials.

Summary of the Invention

According to the present invention, a novel metalloproteinase inhibitor (MI), as well as analogs of MI, are provided. Also provided are DNA sequences coding for all or part of MI, vectors containing such DNA sequences, and host cells transformed or transfected with such vectors. Also comprehended by the invention are methods of producing recombinant MI, and methods of treating disorders. Additionally, pharmaceutical compositions including MI and antibodies specifically binding MI are provided.

Brief Description of the Drawings

Note that in all Figures showing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), numbered marks at the left represent migration positions of standards having molecular weights of 10^3 times the indicated numbers. These markers were phosphorylase b (M_r of 97,400), bovine serum albumin (BSA; M_r of 66,200), ovalbumin (M_r of 42,700) carbonic anhydrase (M_r of 31,000), soybean trypsin inhibitor (M_r of 21,500), and lysozyme (M_r of 14,400). The standards were always reduced, even when some other samples run on the same gel were unreduced.

Figure 1 shows the cDNA sequence and amino acid sequence of bovine metalloproteinase inhibitor.

Figure 2 shows the cDNA sequence and amino acid sequence of human metalloproteinase inhibitor.

Figure 3 shows anion exchange chromatography used in purification of bovine peak I-derived metalloproteinase inhibitor (MI).

Figure 4 shows chromatofocusing used in purification of bovine peak I-derived metalloproteinase inhibitor (MI).

Figure 5 shows SDS-PAGE of bovine peak I-derived metalloproteinase inhibitor (MI) and peak II-derived metalloproteinase inhibitor. A is SDS-PAGE with silver-staining, B is SDS-gelatin PAGE, and C is SDS-PAGE with immunoblotting.

Figure 6 shows effect of EDTA and of bovine speak I-derived metalloproteinase inhibitor (MI) on gelatinolytic proteinases run on SDS-gelatin PAGE.

Figure 7 shows autoradiography illustrating effect of bovine peak I-derived metalloproteinase inhibitor (MI) on specific collagen cleavage.

Figure 8 shows a diagram of plasmid constructions made for expression of recombinant human metalloproteinase inhibitor in Escherichia coli.

Figure 9 shows a synthetic DNA fragment constructed for use in the expression of recombinant human metalloproteinase inhibitor in E. coli, containing a ribosome binding site, an initiation methionine codon, and codons for the first 42 amino acids of the mature protein.

Figure 10 shows vectors used for expression of recombinant human metalloproteinase inhibitor in yeast.

Figure 11 shows vectors used for isolation of yeast secretion mutants.

DNA sequences of the invention are also suitable materials for use as labeled probes in isolating human genomic DNA encoding MI and related proteins as well as cDNA and genomic DNA sequences of other mammalian species. DNA sequences may also be useful in various alternative methods of protein synthesis (e.g., in insect cells) or in genetic therapy in humans and other mammals. DNA sequences of the invention are expected to be useful in developing transgenic mammalian species which may serve as eucaryotic "hosts" for production of MI and MI products in quantity. See, generally, Palmiter et al., Science 222, 809-814 (1983).

The present invention provides purified and isolated polypeptide products having part or all of the primary structural conformation (i.e., continuous sequence of amino acid residues) and one or more of the biological properties (e.g., immunological properties and in vitro biological activity) and physical properties (e.g., molecular weight) of naturally-occurring MI including allelic variants thereof. The term "purified and isolated" as used herein means substantially homogeneous or purified to apparent homogeneity (e.g., one band by SDS-PAGE). These polypeptides are also characterized by being the natural purified product, or the product of chemical synthetic procedures or of procaryotic or eucaryotic host expression (e.g., by bacterial, yeast, higher plant, insect and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis. The products of expression in typical yeast (e.g., Saccharomyces cerevisiae) or procaryote (e.g., E. coli) host cells are free of association with any mammalian proteins. The products of expression in vertebrate (e.g., non-human mammalian (e.g. COS or CHO) and avian) cells are free of association with any human proteins. Depending upon the host employed, polypeptides of the invention may be glycosylated with mammalian or other eucaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue (at position -1).

In addition to naturally-occurring allelic forms of MI, the present invention also embraces other MI products such as polypeptide analogs of MI and fragments of MI. Following the procedures of the above-noted published application by Alton et al. (WO 83/04053), one can readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of cDNA and genomic genes may be readily accomplished by well-known site-directed mutagenesis techniques and employed to generate analogs and derivatives of MI. Such products would share at least one of the biological properties of MI but may differ in others. As examples, projected products of the invention include those which are foreshortened by e.g., deletions; or those which are more stable to hydrolysis (and, therefore, may have more pronounced or longer lasting effects than naturally-occurring); or which have been altered to delete one or more potential sites for O-glycosylation (which may result in higher activities for yeast-produced products); or which have one or more cysteine residues deleted or replaced by, e.g., alanine or serine residues and are potentially more easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine and bind more or less readily to target proteins or to receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within MI, which fragments may possess one activity of MI (e.g., receptor binding) and not others (e.g., metalloproteinase inhibiting activity). It is noteworthy that activity is not necessary for any one or more of the products of the invention to have therapeutic utility [see, Weiland et al., Blut 44, 173-175 (1982)] or utility in other contexts, such as in assays of MI antagonism. Competitive antagonists may be quite useful in, for example, cases of overproduction of MI.

Of applicability to MI fragments and polypeptide analogs of the invention are reports of the immunological activity of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically active animals. See, e.g., Lerner et al., Cell 23, 309-310 (1981); Ross et al., Nature 294, 654-656 (1981); Walter et al., Proc. Natl. Acad. Sci. USA 77, 5197-5200 (1980); Lerner et al., Proc. Natl. Acad. Sci. USA, 78 3403-3407 (1981); Walter et al., Proc. Natl. Acad. Sci. USA 78, 4882-4886 (1981); Wong et al., Proc. Natl. Acad. Sci. USA, 79 5322-5326 (1982); Baron et al., Cell 28, 395-404 (1982); Dressman et al., Nature 295, 185-160 (1982); and Lerner, Scientific American 248, 66-74 (1983). See, also, Kaiser et al. [Science 223, 249-255 (1984)] relating to biological and immunological activities of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation.

The present invention also includes that class of polypeptides coded for by portions of the DNA

of unpurged bone marrow cells will alleviate the need for developing expensive purging techniques.

Diagnostically, correlation between absence of MI production in a tumor specimen and its metastatic potential is useful as a prognostic indicator as well as an indicator for possible prevention therapy.

Tumors may also become more or less encapsulated or fibrotic due to increased collagen deposition (or inhibition of breakdown) by both cancer cells and/or surrounding normal cells. Increased encapsulation promoted by MI aids in clean tumor excision.

Other pathological conditions in which excessive collagen degradation may play a role and thus where MI can be applied, include emphysema, Paget's disease of bone, osteoporosis, scleroderma, pressure atrophy of bone or tissues as in bedsores, cholesteatoma, and abnormal wound healing. MI can additionally be applied as an adjunct to other wound healing promoters, e.g., to modulate the turnover of collagen during the healing process.

MI also plays a role in the hematopoietic processes based on its erythroid potentiating activity (i.e., stimulation of differentiation of early erythroid progenitors), and thus MI is useful in the treatment of various anemias.

In addition MI has application in the treatment of immunological disorders such as autoimmune diseases (e.g., rheumatoid arthritis, multiple sclerosis) based upon its ability to suppress B-cell differentiation as determined by the method of Pisko et al. [J. Immunol. 136, 2141-2150 [1986)].

Based on its ability to inhibit connective tissue degradation and to inhibit proliferation of capillary endothelial cells, MI and/or TIMP has application in cases where inhibition of angiogenesis is useful, e.g., in preventing or retarding tumor development.

The subject invention also relates to antibodies specifically binding metalloproteinase inhibitor. Example 6 below describes the production of polyclonal antibodies. A further embodiment of the invention is monoclonal antibodies specifically binding MI. In contrast to conventional antibody (polyclonal) preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies are useful to improve the selectivity and specificity of diagnostic and analytical assay methods using antigen-antibody binding. A second advantage of monoclonal antibodies is that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may be prepared from supernatants of cultured hybridoma cells or from ascites induced by intra-peritoneal inoculation of hybridoma cells into mice. The hybridoma technique described originally by Köhler and Milstein [Eur. J. Immunol. 6, 511-519 (1976)] has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

EXAMPLE 1

Purification/Characterization of Metalloproteinase Inhibitors From Bovine Aortic Endothelial Cell Conditioned Medium.

1. Conditioned medium.

Bovine aortic endothelial cells (cell line $NCACl_2$; De Clerck et al., Cancer Research, supra) were cultured in Eagle's Minimum Essential Medium (MEM) containing fetal bovine serum (2%, v/v) supplemented with MITO+ serum extender (2%, v/v; Collaborative Research, Inc., Bedford, MA), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells between passage 10 and 20 were grown in 800 cm^2 roller bottles (Costar). For conditioning, cultures at 80-90% confluence were washed 3 times with serum-free medium over 4 to 5 h and then incubated in the presence of fresh serum-free medium for 48 h. The medium was collected, centrifuged at 5,000 x g for 10 min at 4°C and kept at 4°C after addition of sodium azide (0.02%, w/v). After the conditioning, cells were trypsinized, diluted 1:4 with medium, and grown to 80% confluence for reconditioning.

2. Inhibition assays.

above, using aliquots (15 µl) from the indicated fractions. Fractions collected during sample application are not shown; no inhibitor activity was present in these fractions.

2. Chromatofocusing.

The fractions from the Mono Q column runs that contained inhibitor activity were combined and the pool (12 ml) was dialyzed against 25 mM bis Tris-HCl, 1 mM $CaCl_2$, 0.05% (w/v) Brij-35, pH 7.4 and applied to a Mono P chromatofocusing column (Pharmacia; 4 ml) equilibrated in the same buffer, at room temperature. No inhibitor activity was present in fractions collected during sample application. Elution of bound material was accomplished with a pH gradient generated by applying a solution of polybuffer 74 (Pharmacia) diluted ten-fold and adjusted to pH 4 with HCl. Fractions of 1 ml were collected at a flow rate of 0.5 ml/min, immediately brought to 50 mM in Tris-HCl by addition of 50 µl of 1M Tris-HCl, pH 7.5, and further titrated to pH 7.5 by addition of 2 M Tris base. The elution profile is shown in Figure 4. Aliquots (5 µl) from the indicated fractions were measured for inhibitor activity as described in section 2 above. There is a peak of inhibitor activity eluting at about pH 5.5 (fractions 21-27), plus activity eluting later (fractions 30-45). These latter fractions were pooled, dialyzed against the Mono P starting buffer, and rechromatographed on the Mono P column as for the original sample. Recovered activity was redistributed between an earlier-eluting peak (about pH 5.5) and later-eluting region, with the early peak representing about one-third of the recovered activity. The later-eluting material from this second Mono P run was chromatographed again, with similar redistribution of the activity. The early-eluting fractions (pH 5.5 peak) from all three Mono P column runs were combined.

3. Gel filtration.

The combined pool from the Mono P column runs (15 ml) was concentrated to 3 ml using Amicon Centricon 10 units centrifuged at 5000 x g in a fixed-angle rotor. The concentrated sample was then applied to a Sephadex G-100 gel filtration column (1.5 x 94 cm) equilibrated with 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5. Fractions of 2.1 ml were collected, at a flow rate of 5 ml/h. A single peak of inhibitor activity was recovered, eluting with an apparent molecular weight of 24,000 relative to molecular weight markers used for column calibration (myoglobin, M_r of 17,000; ovalbumin, M_r of 44,000; gamma-globulin, M_r of 158,000), and having a specific activity of about 1,550 U/mg.

In the purification of peak I-derived inhibitor, it should be noted that the second Sephadex G-100 gel filtration step was useful because the material at this stage behaved with an apparent molecular weight of 24,000 rather than the 70,000-75,000 true for peak I activity on the initial Sephadex G-100 column.

A summary of the purification for peak I-derived material is shown in Table 1.

Table I. (See following page.) Purification of two metalloproteinase inhibitors from bovine aortic endothelial cells. After steps 1 and 2, the inhibitors were separately purified as indicated. Recovery and degree of purification were calculated separately for the two inhibitor preparations, assigning values of 100% and 1, respectively, for each of the step 2 Sephadex G-100 peaks.

Footnotes:

[a]Determined by the method of Bradford [Anal. Bioch. 72, 248-254 (1976)] using BSA as standard, except where indicated otherwise.

[b]Estimate, based on intensity of silver-stained bands after SDS-PAGE.

d. Peak II purification.

1. Heparin-Sepharose.

The peak II material from gel filtration (465 ml; 3b above) was dialyzed against 25 mM sodium cacodylate-HCl, 10 mM $CaCl_2$, 0.05% (w/v) Brij-35, pH 7.5 and chromatographed on a heparin-Sepharose column equilibrated with this buffer. After column washing, elution of bound material was achieved with a linear gradient to 1 M NaCl in the same buffer [see De Clerck, Arch. Biochem. Biophys. 265, 28-37 (1988)].

2. Anion exchange.

The active fractions from heparin-Sepharose were combined (total volume 7.5 ml) and dialyzed against 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.5. The material was then divided and applied in two separate chromatographic runs to a Mono Q column as described above (c.1). Eighty to 90% of the recovered activity was present in fractions collected during sample application (unbound), and represented highly-purified peak II-derived inhibitor material with a specific activity of about 1,780 U/mg. The remainder of the activity eluted early (about 0.065 M NaCl) in the salt gradient.

A summary of the purification for peak II-derived material is shown in Table 1.

4. Characterization of peak I-derived and peak II-derived inhibitors.

a. SDS-PAGE was carried out by the method of Laemmli [Nature 227, 680-685 (1970)]. Stacking gels contained 4% (w/v) acrylamide and separating gels contained 12.5% (w/v) acrylamide. Samples were prepared under reducing or non-reducing conditions, that is, with or without 2-mercaptoethanol present in the treatment buffer. After electrophoresis, gels were subjected to silver-staining [Morrissey, Anal. Biochem. 117, 307-310 (1981)] or immunoblotting [Burnette, Anal. Biochem. 112, 195-203 (1981)].

1. Peak I-derived inhibitor.

The active fractions from the Sephadex G-100 column (c.3 above) all contained a fairly sharp major band evident upon SDS-PAGE with silver-staining, migrating with apparent molecular weight of 24,000-28,000 (reduced) and 19,000-22,000 (unreduced). This band was also evident in active fractions from the step that preceded (c.2 above; Mono P). Such co-elution of activity and material banding at this position is consistent with the conclusion that the band represents active protein. This band can be seen in Figure 5A, lanes 1 and 2. For these gels, 100 µl of the final gel filtration pool (c.3 above; Table 1, step 2.1.3) was loaded; lane 1, reduced and lane 2, unreduced. Note that the difference in migration for the reduced and unreduced material probably reflects the presence of intrachain disulfide bonds in the unreduced case.

Figure 5C, lane 1 shows the result of SDS-PAGE with immunoblotting for a similar sample (250 mU; unreduced). The primary antibody in the immunoblotting was a rabbit polyclonal antibody against TIMP from bovine vascular smooth muscle cells (De Clerck, Arch. Biochem. Biophys., supra) used at 1:500 dilution. (The secondary antibody used for detection was a goat anti-rabbit antibody conjugated with horseradish peroxidase.) No bands were visualized with the use of this primary antibody. Note that Figure 5C, lane 3 shows that TIMP from bovine vascular smooth muscle cells (160 mU; unreduced) is visualized with the use of this antibody.

2. Peak II-derived inhibitor.

SDS-PAGE with silver-staining is shown in Figure 5A, lanes 3 (reduced) and 4 (unreduced) for the unbound material from Mono Q chromatography (d.2 above; Table 1, step 2.2.2; 75 µl loaded). The staining material migrates over a fairly broad region representing a molecular weight range of 30,000-34,000 (reduced) and 27,000-31,000 [unreduced).

TABLE 2

Effect of peak I-derived inhibitor on various collagenases

Enzyme	Substrate	Peak I-derived inhibitor amount (mU)	Inhibition (%)
Type I collagenase[a]	^{14}C-labeled type I collagen[d]	50	100
Gelatinase[a]	^{14}C-labeled type I collagen, heat denatured[e]	50	90
Bacterial collagenase[b]	^{14}C-labeled type I collagen[d]	200	0
Type IV collagenase[c]	^{14}C-labeled type IV collagen[d]	200	66

[a]Trypsin-activated conditioned medium from TPA-treated rabbit synovial fibroblasts (40 mU; see Example 1, section 2).

[b]Form III from Clostridium histolyticum (34 mU) (Advance Biofacture Corp., Lynbrook, NJ).

[c]Trypsin-activated conditioned medium from mouse reticulum cell sarcoma cell line (50 µl of 104-fold concentrated medium; see De Clerck, Arch. Biochem. Biophys., supra).

[d]See Example 1, section 2.

[e]Heat-denatured at 60°C for 20 min.

e. SDS-PAGE of ^{14}C-labeled collagen degradation products generated by type I (classical) collagenase in the absence and presence of the peak I-derived inhibitor is shown in Figure 7. ^{14}C-Labeled type I collagen (30,000 cpm) was incubated under the conditions described for inhibition assays in Example 1, section 2, with various additions, at 22°C

TABLE 3

Stability of peak I-derived and peak II-derived inhibitors. Inhibitor samples (2 U/ml) were treated as indicated prior to being tested for residual anticollagenase activity determined from dose-inhibition curves.

Treatment	Conditions	Loss of inhibitory activity (%)	
		Peak I-derived inhibitor	Peak II-derived inhibitor
(A) Heat[a]	37 °C	0	11
	50 °C	0	11
	80 °C	9	30
	100 °C	59	44
(B) Trypsin[b]			
	1:1	10	0
	10:1	28	21
	50:1	100	100
(C) Acid pH 4.5, 22 °C, 1 h		0	0
(D) Reduction-alkylation[c]		100	100

[a]Inhibitor samples were incubated at indicated temperatures for 1 h. Loss of inhibitory activity was calculated in comparison with an untreated sample.
[b]Samples were incubated at indicated trypsin:inhibitor ratios (w:w) for 1 h at 37 °C. The reaction was then blocked with five-fold weight excess of soybean trypsin inhibitor. Loss of activity was deter ined in comparison with a sample incubated at 37 °C for 1 h in the presence of a trypsin-soybean trypsin inhibitor mixture.
[c]Samples were reduced by the addition of 2-mercaptoethanol (20 mM) for 16 h at 4 °C and alkylated with iodoacetamide (20 mM) at 30 °C for 1 h. Loss of activity was determined by comparison with samples ncubated at the same temperatures. 2-Mercaptoethanol and iodoacetamide did not affect collagenase activity.

EXAMPLE 2

Amino-Terminal Amino Acid Sequence Analysis of Peak I-Derived Inhibitor and Peak II-Derived Inhibitor: Amino Acid Composition Analysis of Peak I-Derived Inhibitor.

Peak I-derived inhibitor (4.8 ml; Table 1, step 2.1.3) was concentrated and introduced into 50 mM ammonium bicarbonate, pH 7.8 using an Amicon Centricon 10 ultrafiltration unit. The sample was spotted onto a glass fiber disc on a sequencer cartridge, which had been pre-cycled with polybrene. The glass fiber disc containing sample was dried under a stream of N_2. Amino-terminal amino acid sequence analysis was performed according to published methods [Hewick et al., J. Biol. Chem. 256, 7990-7997 (1981)] with Applied Biosystems Model 477 protein sequencer using a standard program provided by Applied Biosystems (Foster City, CA). The released phenylthiohydantoin (PTH)-amino acids were analyzed by a Model 120 on-line PTH-amino acid analyzer using a Brownlee reverse phase C-18 column. The chromatog-

TABLE 5

Amino-terminal sequence of bovine peak II-derived inhibitor

```
 1   2   3   4   5   6   7   8   9   10  11  12  13
(Cys)-Thr-(Cys)-Val-Pro-Pro-His-Pro-Gln-Thr-Ala-Phe-(Cys)-

 14  15  16  17  18  19  20  21  22  23  24  25  26
Asn-Ser-Asp-Val-Val-Ile-Arg-Ala-Lys-Phe-Val-Gly-Thr-

 27  28  29  30   31  32  33  34  35  36  37  38  39
Ala-Glu-Val-(Asn)-Glu-Thr-Ala-Leu-Leu-Tyr-Arg-Tyr-Leu-

 40  41  42  43  44    45  46  47  48   49
Ile-Lys-Met-[Leu]-Lys-Met-Pro-Ser-[Gly]-Phe---
```

The initial yield was approximately 280 pmol and the average repetitive yield was 92%. Residues 1, 3 and 13 were assigned as cysteines for the reasons described above. Residue 30 was also not recovered and was assigned as asparagine since the subsequent sequence

```
(Asn-Glu-Thr...)
 30  31  32
```

would be consistent with an Asn-linked glycosylation site. The assignments at positions 43 and 48 (in brackets) were made with less than full confidence.

Based on these various analyses (Examples 1 and 2), the peak II-derived material is almost certainly bovine TIMP. Human TIMP is very well characterized and has been cloned (Docherty et al., Nature, supra; Carmichael et al., Proc. Natl. Acad. Sci. USA, supra). Comparing the amino-terminal sequences of human TIMP and the peak II-derived material, the homology over the first 29 residues is 93% and the homology over the first 49 residues is 80% (see Table 6). In addition the isolated bovine peak II-derived material shares many of the biochemical properties of TIMP, i.e., behavior in various purification steps, mobility on SDS-PAGE, and recognition by antibody to bovine smooth muscle TIMP in SDS-PAGE with immunoblotting (Example 1).

The peak I-derived material (MI) is clearly distinct from TIMP (Table 6) in amino acid sequence, but does have homology to TIMP. Homology over the first 29 residues is 65%, and homology over the first 45 residues is 47%. The molecules have different chromatographic behaviors, different mobilities on SDS-PAGE, and antibody to bovine smooth muscle TIMP does not visualize the peak I-derived material in immunoblots after SDS-PAGE (Example 1). This novel peak I-derived inhibitor is designated metalloproteinase inhibitor (MI).

Over the first 45 residues, the peak I-derived and peak II-derived bovine inhibitors have 51% homology to each other.

TABLE 7

Amino acid composition analysis of bovine peak I-derived inhibitor (MI)			
	Residues per molecule		
Amino acid	Average value	Integral value	"Actual" value[c]
Lys	15.5	16	17
His	3.6	4	4
Arg + Thr[a]	15.9	16	6 + 6 = 12
Asx[1]	22.9	23	22
Ser	10.9	11	10
Glx[1]	19.4	19	19
Pro	8.1	8	12
Gly	14.2	14	13
Ala	15	15	16
Val	9	9	8
½-Cys	nd[b]		(12)
Met	3.1	3	5
Ile	14.6	15	19
Leu	10.8	11	7
Tyr	7.1	7	7
Phe	7.7	8	7
Trp	nd[b]		(4)
	178	179	178 + 12 + 4 = 194

[a] Arg and Thr were not separable by the method used.
[b] Not determined.
[c] Values from gene-encoded sequence of mature bovine MI polypeptide; see Example 3, Figure 1.

EXAMPLE 3

Cloning of the Bovine and Human Metalloproteinase Inhibitor Genes.

The amino-terminal amino acid sequence for the bovine metalloproteinase inhibitor was determined as described above and 3 probes were designed and manufactured on DNA synthesizers (Applied Biosystems models 380A and 380B) for hybridization to the sense strand of DNA (or to the mRNA). The first probe was designed as a long nondegenerate probe by the method of Lathe [J. Mol. Biol. 183, 1-12 (1985)] to recognize the region corresponding to amino acids 4 to 19 and is as follows:

5′ GAT CAC AAT GTC AGC ATT GCA GAA GGC CTG CTG GGG ATG CAC AGG 3′

The second and third probes were designed as degenerate probes incorporating inosine bases at positions of 4-fold degeneracy. The second probe recognizes the region corresponding to amino acids 21 to 30 and is as follows:

```
                (T)   (T)   (T)                 (T)
5' GTC IAC (C)TC (C)TT (C)TT GTT IAC IGC (C)TT IGC 3'
```

The parentheses indicate the incorporation of two bases, leading to multiple oligonucleotides in the probe

probe 1

5' CGG GTC CTC GAT GTC CAG AAA CTC CTG CTT GGG GGG TGC TGC
TCC GCG GTA 3'

probe 2

5' GAA CTT GGC CTG GTG TCC GTT GAT GTT CTT CTC CGT GAC CCA
GTC CAT CCA 3'

probe 3

5' GCA CTC ACA GCC CAT CTG GTA CCT GTG GTT CAG GCT CTT CTT
CTG GGT GGC 3'

probe 4

5' GGG GTT GCC GTA GAT GTC GTT GCC AGA GTC CAC CTC CTT CTT
ATT GAC TGC 3'

A λgtII cDNA library made with mRNA isolated from human heart tissue (fetal aorta) was purchased from CLONTECH Laboratories, Inc. Approximately 10^6 phage were plated onto eight 23 x 23 cm square plates with the host bacterial strain, Y1090. Two lifts from each plate were made onto GeneScreen Plus hybridization transfer membranes. One set of membranes was hybridized with a mixture of ^{32}P-phosphorylated probes 1 and 2, and the second set of membranes was hybridized with a mixture of ^{32}P-phosphorylated probes 3 and 4, using the hybridization and wash conditions described above. Three clones hybridized to both sets of probes and these clones were rescreened until isolated plaques were obtained. Mini λ phage DNA preps were made as described above and restriction endonuclease digests were performed on the DNAs. The three clones were of similar but different lengths so one of the clones was subcloned from λgtII into M13 mp9 from EcoRI to EcoRI in both orientations. This EcoRI fragment was subsequently cloned from M13 mp9 into pUC 19 to generate pUC HMI. The original clones in M13 mp9 and additional overlapping restriction fragments cloned from pUC HMI into M13 mp vectors in both orientations were sequenced using the dideoxy method of Sanger (Proc. Natl. Acad. Sci. USA, supra). The sequence of the human metalloproteinase inhibitor gene is shown in Figure 2. It, like the bovine metalloproteinase inhibitor gene, codes for a protein of 194 amino acids with a leader sequence of an additional 26 amino acids. The two genes code for different amino acids at 11 of the 194 residues corresponding to the mature protein. The molecular weight of the mature human MI polypeptide, based on the gene-encoded sequence in Figure 2, is 21,730.

EXAMPLE 4

Expression of Recombinant Human Metalloproteinase Inhibitor in E. coli.

The mature human metalloproteinase inhibitor protein was expressed in E. coli by utilizing an NcoI site at amino acid 1 of the leader sequence, a BamHI site at amino acid 42 of the mature protein, and a StuI site 3 nucleotides downstream from the termination codon. The fragment from NcoI to StuI was first cloned into an expression vector, pCFM 1156 pL, from NcoI to SstII (which had been blunted at the SstII site using the Klenow fragment of DNA polymerase I) to generate p1156 HMI1 (Figure 8). The plasmid pCFM 1156 pL can be derived from plasmid pCFM 836 (see U.S. Patent No. 4,710,473 hereby incorporated by reference), by destroying the two endogenous NdeI restriction sites by end filling with T4 polymerase enzyme followed by

active MI requires procedures for solubilization, purification, folding, and oxidation (disulfide formation) of the inclusion body MI. An example of such procedures is given below.

About 400 grams (wet weight) of cell paste of E. coli strain FM5 harboring plasmid p1156 HMI2, grown as indicated in Example 4, was suspended in 1.5 liters of H_2O. The material was passed through a Manton-Gaulin homogenizer three times and then centrifuged for 45 min at about 4,000 x g at 4°C. The supernatent was poured off and discarded. The pellets were resuspended in 1.5 liters H_2O (4°C) and centrifuged as above. The supernatant was poured off and discarded. The pellets were resuspended in 120 ml H_2O and then diluted ten-fold with 20 mM Tris-HCl, pH 9.5. The pH was adjusted to 11.5 (using 1 N NaOH), and the mixture was left on ice for 15 min, and then centrifuged for 30 min at 11,300 x g at 4°C. The supernatent was diluted four-fold with 20 mM Tris-HCl, pH 9.5. The pH was adjusted to 10-10.5 (with 1 N NaOH) and the mixture was stirred overnight at room temperature.

The pH of the mixture was lowered to 8.5 (using 1 N HCl) and the mixture was then loaded onto a DEAE-Sepharose Fast Flow (Pharmacia) ion exchange column (150 ml column volume) equilibrated in 20 mM Tris-HCl, pH 8.5. Bound material was eluted with a 2 liter gradient from 0 to 0.3 M NaCl in the Tris-HCl buffer. Fractions of 12 ml were collected at a flow rate of 8 ml/min. Aliquots (25 µl) of collected fractions were subjected to SDS-PAGE (15%, w/v, acrylamide; unreduced) with Coomassie blue staining. Fractions 38-54, which contained a fairly sharp band (M_r about 22,000-23,000) corresponding to the MI polypeptide, were pooled (202 ml). Material thought to also represent the MI polypeptide, but having a slightly lower mobility and banding less sharply on SDS-PAGE, eluted later in the gradient and was not included in the pool.

The pooled material from DEAE-Sepharose Fast Flow was concentrated to 30 ml using an Amicon stirred cell (with YM5 membrane). The pH was adjusted to 5.4 (using 50% acetic acid) and the mixture was dialysed against 20 mH sodium acetate, pH 5.4. The material was then diluted with H_2O to a final volume of 45 ml and applied to a CM-Sepharose Fast Flow (Pharmacia) ion exchange column (1 ml column volume) equilibrated in 20 mM sodium acetate, pH 5.4. Bound material was eluted using a 20 ml gradient from 0 to 0.4 M NaCl in the sodium acetate buffer. Fractions of 1 ml were collected at a flow rate of 0.1 ml/min. Aliquots (10 µl) of the fractions were analyzed by SDS-PAGE as above and those containing MI [fractions 11-18 (8 ml)] were pooled and then loaded directly onto a Sephacryl S-200 HR gel filtration column (300 ml column volume) equilibrated in phosphate-buffered saline (PBS). Fractions of 4 ml were collected at a flow rate of 20 ml/h. Aliquots (20 µl) of the fractions were again analyzed by SDS-PAGE as above. Fractions 54-60 contained MI; to maximize purity, only fractions 56-59 were pooled (16 ml). Purity of MI in the pool, estimated by SDS-PAGE, was greater than 90% as judged by visual inspection of gels after SDS-PAGE with Coomassie blue staining. Total pro-in in the pool, measured by the method of Bradford (Anal. Biochem., supra) using BSA as standard, was about 8 mg. Inhibitory activity of this material was about 424 U/ml (specific activity about 865 U/mg) measured by the type I collagenase inhibition assay described in Example 1, section 2. Inhibitory activity of E. coli-derived human MI was also demonstrated in several other ways (Example 11).

A sample of the human MI preparation described (about 6.5 µg) was subjected to amino-terminal amino acid sequencing through 18 cycles, using the methods described in Example 2. The initial yield was 135 pmol and the repetitive yield was 94%. The major sequence obtained exactly matched that predicted from the nucleotide sequence for the mature human MI gene (Example 3; Figure 2).

The material is purified to apparent homogeneity using methods such as that described in Example 1 for bovine MI or other methods evident to those skilled in the art.

EXAMPLE 6

Generation of Rabbit Polyclonal Antisera to Human Metalloproteinase Inhibitor.

Two types of preparation of metalloproteinase inhibitor were used for generation of rabbit polyclonal antisera. The first (used for injections on days 1, 7 and 21) was prepared as follows. About 14 g (wet weight) of cell paste from E. coli strain FM5 harboring plasmid p1156 HMI2 (Example 3) was suspended in 50 ml H_2O and passed twice through a French Press device. The pellet fraction obtained by centrifugation was resuspended in a final volume of 10 ml containing sodium sarkosyl (2%, w/v), Tris-HCl (50 mM), dithiothreitol (50 mM) with pH of 8.5, and incubated at 50°C for 10-15 min and room temperature for 2 h, for solubilization of MI. After centrifugation of this mixture, a supernatant fraction (7.2 ml) containing MI was

I. pUC119 with deletion of HindIII, SalI, SstI and SmaI sites: pUC119 was digested with HindIII plus SalI, followed by S1 nuclease treatment to generate blunt ends, then ligation. The resulting plasmid was further digested with SstI plus SmaI, followed by S1 nuclease treatment, then ligation, resulting in deletion of HindIII, SalI, SstI and SmaI sites. An expression casette was then introduced into the remaining unique BamHI site.

II. The expression casette consists of the following:

(i) a 675 bp HindIII to BamHI fragment containing the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (GPD-P) [Bitter et al., Gene 32, 263-278 (1984)], where the HindIII site was removed and a BamHI site was added. This was accomplished by digestion with HindIII followed by end-filling with the Klenow fragment of DNA polymerase I. The DNA fragment containing the end-filled HindIII site was blunt-end ligated into the SmaI site of pUC19.

(ii) A GPD-α-factor linker

```
(Sau3A)                  met arg phe pro ser ile phe thr ala
GATCACACATAAATAAACAAAATG AGA TTT CCT TCA ATT TTT ACT GCA
   TGTGTATTTATTTGTTTTAC TCT AAA GGA AGT TAA AAA TG (PstI)
```

(iii) A 218 bp PstI to HindIII fragment containing the α-factor pre-pro leader sequence from pαC3 [Zsebo et al., J. Biol. Chem. 261, 5858-5865 (1986); Bitter et al., Methods in Enzymol. 153, 516-544 (1987)].

(iv) A linker for joining the α-factor pre-pro leader to the α-factor terminator sequence such as:

```
HindIII SphI SstI SmaI XhoI BglII                (SalI)
AGCTTGCATGCGAGCTCCCCGGGCTCGAGATCTGATAACAACAGTGTAGATGTAACAAAA
   ACGTACGCTCGAGGGGCCCGAGCTCTAGACTATTGTTGTCACATCTACATTGTTTTAGCT
```

(v) An α-factor terminator sequence on an approximately 250 bp SalI to BamHI fragment from pαC3, with the SalI site being destroyed after joining to the linker in (iv).

The α-factor-MI gene fusion was accomplished by digesting pUC119αG4 with HindIII and SmaI followed by ligation with the synthetic DNA linker and the MI DNA fragment. The resultant plasmid pUC119αG4-HMI depicted in Figure 10A contains a yeast glyceraldehyde phosphate dehydrogenase promoter (GPD-P) followed successively by the α-factor pre-pro leader from the yeast MFα1 gene, the synthetic DNA linker above, the human metalloproteinase inhibitor gene DNA segment and α-factor transcription terminator. The 1800 bp BamHI DNA fragment containing the elements above was isolated from pUC119αG4 by a partial digest with BamHI and inserted into the BamHI site of the yeast-E. coli shuttle vector pYE3 resulting in the plasmid pYE3αG4-HMI (Figure 10C).

The plasmid pYe3 is shown in Figure 10B and consists of the following:

I. Yeast 2μ (B form) plasmid in pGT41 [Tschumper et al., Gene 23, 221-232 (1983)] where the 2500 bp BamHI to SalI LEU 2 gene segment was deleted by digestion with BamHI plus SalI, and this treatment was followed by mungbean nuclease treatment to generate blunt ends, followed by ligation.

II. A polylinker, whose sequence is shown below, was inserted into a EcoRI site of the modified 2μ plasmid in (I) as shown in Figure 10B.

```
AATTC GATATC GAT GGTACC CGG GATCC GTCGAC AGATCT G
   G CTATAG CTA CCATGG GCC CTAGG CAGCTG TCTAGA CTTAA
EcoRI EcoRV ClaI KpnI SmaI BamHI SalI   BglII  EcoRI
```

III. A 852 BglII to EcoRI fragment containing the TRP 1 gene [Tschumper et al. Gene 10, 157-166 (1980)] inserted into the BglII and EcoRI sites of the polylinker in (II).

The plasmid pYE3αG4-HMI was grown in E. coli strain DH5α, the plasmid DNA was isolated and the DNA was transformed into the S. cerevisiae yeast strain EG45*. Other yeast host cells can also be used as will be apparent to those skilled in the art.

this band was present at about 25 to 50 mg per liter of supernatant. The M_r 26,000 band was not observed in control fermentor supernatants. The M_r 26,000 band had the same mobility on SDS-PAGE as MI purified from bovine endothelial cell conditioned medium (Example 1). By SDS-PAGE with silver-staining performed on aliquots (10 µl) of MI-containing yeast supernatants in unreduced conditions, the M_r 26,000 band was absent, and there was instead an M_r 22,000-23,000 band. Material represented by the M_r 22,000-23,000 band was present at about 2-5 mg per liter of supernatant, and was not seen in control supernatants. To demonstrate that the M_r 26,000 (reduced) and M_r 22,000-23,000 (unreduced) bands represented human MI, a polyclonal antibody raised in rabbits against human MI produced in E. coli (Example 6) was used. SDS-PAGE with immunoblotting (Burnette, Anal. Biochem., supra) was performed using this antibody preparation and a Vectastain ABC kit (Vector laboratories) containing biotinylated anti-rabbit immunoglobulin, avidin, and biotinylated horseradish peroxidase. Immunoreactive bands were seen for supernatants from the yeast strain transfected with the MI gene-containing plasmid (10 µl loaded) and not for control supernatants. [M_r 26,000 and M_r 18,000 bands were present for reduced samples and M_r 22,000-23,000 band for unreduced samples. The M_r 18,000 (reduced) band is presumed to be a proteolytic breakdown fragment of MI.] The antibody also reacted in immunoblots with MI purified from bovine enthothelial cell conditioned medium (350 mU) and with E. coli-produced human MI (0.3 µg) which indicates that the bands observed in yeast supernatants did in fact represent human MI.

EXAMPLE 9

Expression of Human Metalloproteinase Inhibitor in Chinese Hamster Ovary Cells.

1. Construction of an expression vector.

To generate expression plasmids, the NcoI to EcoRI fragment of pUC HMI (Example 3) containing the intact coding sequence of human MI [including the sequence coding for the 26-amino acid leader Figure 2)] was first subcloned into pCFM 1156, from the NcoI to the EcoRI restriction site to give plasmid p1156 HMINR. The plasmid pCFM 1156 was derived from plasmid pCFM 836 (see U.S. Patent No. 4,710,473 hereby incorporated by reference), by destroying the two endogenous NdeI restriction sites, end filling with T4 DNA polymerase followed by blunt end ligation and substituting the small DNA sequence between the unique ClaI and KpnI restriction sites with the following oligonucleotide:

```
      ClaI                                                KpnI
5'  CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC  3'
3'    TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC      5'
```

The human MI cDNA was retrieved from plasmid p1156 HMINR as a 0.65 kb HindIII to StuI fragment. This fragment was then cloned into the expression vector pDSRα2 to generate plasmid pDSFα2-MI.

Plasmid pDSRα2 has the following important features (following the map in Figure 12 in a clockwise direction):

(a) SV40 early promoter/enhancer and origin of replication; composed of SV40 sequences between PvuII (SV40 nucleotide map coordinate #272) and HindIII (map coordinate #5172) sites. [DNA Tumor Viruses, J. Tooze, ed., Gold Spring Harbor Laboratories, Cold Spring Harbor, NY (1981), pp. 801-804].

(b) A 267 bp fragment containing the "R" element and part of the "U5" sequences of the long terminal repeat (LTR) of human T-cell leukemia virus type 1 (HTLV-1). This fragment maps at the exact 5' end of "R" (position 354) to the Sau3A site in the U5 sequences (position 620) [Seiki et al. Proc. Natl. Acad. Sci. USA 80, 36183622 (1983)].

(c) A fragment composed of SV40 165, 19S splice donor/acceptor signals (map coordinates #502-560 and #1410-1498 joined by an BamHI linker).

The structural organization of the above three segments (a), (b) and (c) is identical to the published vector pCD-SRα [Takebe et al., Mol. Cell. Biol. 8, 466-472 (1988)] with the following modifications: (1) at the 5' end of segment (a), the HindIII site has been destroyed by end-filled ligation done with the Klenow

produced about 1 mg/liter/day on a confluent 100 mm tissue culture dish without amplification.

5. Amplification of expression.

MI expression by transfected CHO cell clones was amplified by the use of methotrexate [Kaufman and Sharp, J. Mol. Biol. 159, 601-621 (1982)] in stages of increased concentration (stages of 10 nM, 30 nM, 100 nM, and 300 nM methotrexate). Transfected clones subjected to the 10 nM methotrexate amplification stage when grown in roller bottles as described in Example 10, led to conditioned medium containing MI at levels as high as 20-30 mg/liter at the time of the 6-7 day harvesting. After the 300 nM amplification stage, MI levels as high as 50-60 mg/liter could be obtained upon culturing in the roller bottles.

6. Bioactivity assay.

Activity could be detected in transfected CHO cell supernatants by the type I collagenase inhibition assay described in Example 1. Results are given in Example 10 (Table 8).

EXAMPLE 10

Purification of Chinese Hamster Ovary Cell-Expressed Recombinant Human Metalloproteinase Inhibitor.

CHO cells, transfected with the expression vector carrying the human MI gene as described in Example 9 and subjected to the 10 nM methotrexate amplification stage (Example 9), were grown in roller bottles in serum-free medium, as follows. Initially, the cells were grown in spinner flasks in medium containing Dulbecco's modified MEM supplemented with dialyzed fetal calf serum (5%, v/v), glutamine, and non-essential amino acids, plus F12 nutrient medium; the MEM and F12 medium were present at 50:50 (v:v). Cells were then transferred to 850 cm^2 roller bottles (2 x 10^7 cells/bottle) containing the same medium. After three to four days at 37° C, the cell monolayers were washed with PBS and fresh medium (150-200 ml/bottle; as above, but lacking serum) was added. Conditioned medium was harvested 6-7 days later, and replaced with fresh medium again. Six to 7 days later, the additional conditioned medium was harvested.

All subsequent work was done at 4° C unless otherwise indicated. To 20 liters of conditioned medium, sodium azide (final concentration 0.02%, w/v) and the protease inhibitors pepstatin A (final concentration 1 µg/ml) and phenylmethanesulfonyl chloride (PMSF; final concentration 0.6 mM) were added. The medium was concentrated and diafiltered against 1 mM imidazole, 1 M NaCl, pH 7.5 (adjusted with HCl), using a Millipore Pellicon tangential flow ultrafiltration apparatus with 10,000 molecular weight cutoff polysulfone membrane cassette (5 ft^2 membrane area). Pump rate was about 500 ml/min and filtration rate about 100 ml/min. The final volume of recovered sample was 1 liter. This sample was applied to a Chelating Sepharose Fast Flow (Pharmacia) column (400 ml column volume) which had been saturated with Cu^{2+} by passing a solution of $CuSO_4$ over the column, and then equilibrated with the 1 mM imidazole, 1 M NaCl, pH 7.5 buffer. Flow rate was 800 ml/h. After sample application, the column was washed with about 1 liter of the imidazole starting buffer. The MI, which was bound to the column, was then eluted with a linear gradient (20 liters total volume) from the starting buffer to mM imidazole, 1 M NaCl, pH 7.5. Fractions of 420-600 ml were collected and aliquots (0.00033% of fraction volume) were subjected to SDS-PAGE (12.5%, w/v, acrylamide; reduced) with silver-staining. A fraction of 600 ml, representing elution volume 1810-2410 ml in the gradient, contained most of the MI polypeptide (visualized as a band of Mr about 26,000). This fraction was concentrated to about 100 ml using an Amicon stirred cell with an Amicon YM10 membrane, dialyzed against 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.5, and applied to a Q-Sepharose Fast Flow (Pharmacia) ion exchange column (40 ml column volume) equilibrated in the same buffer. Flow rate was 120 ml/h. After sample application, the column was washed with about 100 ml of the starting Tris-HCl buffer. The MI, which was bound to the column, was then eluted with a linear gradient of 0 to 0.5 M NaCl in the starting Tris-HCl buffer (total gradient volume 1200 ml). Fractions of 12.6 ml were collected. Aliquots (1 µl) of the fractions were again analyzed by SDS-PAGE as above, and those containing MI (fractions 25-32 of the gradient) were pooled (100 ml total volume), concentrated to 40 ml using an Amicon stirred cell as above, and applied to a Sephacryl S-200 HR (Pharmacia) gel filtration column (5 x 146 cm) equilibrated with PBS.

Lane 2, peak I-derived inhibitor (MI) from bovine endothelial cells (50 mU);
Lane 3, human MI prepared from E. coli (Example 5; 92 mU);
Lane 4, human MI preparation from E. coli (Example 5; 420 mU);
Lane 5 and 6, buffer only lanes. Noting the dark zones in lanes 1-4, it is apparent that all of the indicated inhibitor preparations, including the recombinant preparations from E. coli, have proteins of the expected molecular weights with inhibitory activity as judged by this method.

The method of SDS-gelatin PAGE with proteinases as samples (Example 1, section 4c) was also used to analyze the E. coli-produced recombinant human MI (Figure 14). In Figure 14, lanes marked "control" were incubated overnight with no inhibitor additions; lanes marked "EDTA" were incubated with 20 mM EDTA present; lanes marked "rMI" were incubated with E. coli-produced human MI (preparation of Example 4, 423 mU/ml). The samples electrophoresed prior to the overnight incubations were: lanes 1, human plasmin, 50 µg; lanes 2, bovine trypsin, 0.3 µg; lanes 3, 5 µl of 100-fold concentrated and APMA-activated conditioned medium from metastatic tumor cells [c-Ha-ras-transfected rat embryo fibroblasts, as source of type IV collagenase; conditioned medium prepared according to Garbisa et al., Canc. Res. 47, 1523-1528 (1987)]; lanes 4, APMA-activated conditioned medium from TPA-treated rabbit synovial fibroblasts (4 mU of collagenase I activity loaded; see Example 1, section 2). It is apparent that the recombinant MI inhibits the type I and type IV collagenases, but does not inhibit plasmin and trypsin (which are not metalloproteinases). EDTA also inhibits the collagenases, as expected.

The recombinant human MI from E. coli also inhibited the specific collagen cleavage characteristic of mammalian collagenases (see Example 1 and Figure 7). Experiments showing this were done essentially as described for Figure 7 in Example 1, using the recombinant human MI (Example 5) at about 2 µg/ml in the incubations. Results were equivalent to those shown in Figure 7 for the bovine MI from endothelial cells.

Human MI from CHO cells had inhibitory activity of about 1537 U/ml (specific activity about 1067 U/mg measured by the type I collagenase inhibition assay described in Example 1, section 2). It is noted that this specific activity, and specific activity in other assays described below, is higher for the recombinant human MI from CHO cells than for that from E. coli. It is expected that this difference is due to the fact that some portion of the polypeptide chains in the E. coli-derived preparation are not in the native conformation and may also have incorrect disulfide bonds; and that one skilled in the art can arrive at procedures for solubilization, folding, oxidation (disulfide bond formation), and purification of E. coli derived human MI such that E. coli-derived human MI would have specific activities comparable to those of CHO cell-derived human MI.

Recombinant human MI from CHO cells also had inhibitory activity as judged by SDS-gelatin PAGE, and by inhibition of the specific collagen cleavage characteristic of mammalian collagenases. In each case the results obtained were similar to those described in the preceding paragraphs for recombinant human MI from E. coli.

2. Inhibition of metalloproteinases secreted by metastatic cells.

Serum-free conditioned medium from c-Ha-ras transfected rat embryo cells (4R), which are highly metastatic [Pozzatti et al., Science 243, 947-950 (1986)], was used as a source of metalloproteinases. The experiments in Figure 15 show that recombinant human MI from CHO cells completely inhibits the degradation of type I and type IV collagen by metalloproteinases secreted by 4R cells. The legend to Figure 15 is as follows. The 4R cells were grown in MEM with penicillin (100 U/ml) and streptomycin (100 µg/ml). The medium was harvested after 24 h of incubation, concentrated 100-fold using an Amicon stirred cell with YM10 membrane, and treated with APMA (1 mM, 37° C, 30 min) to activate metalloproteinases. Aliquots of the activated medium were then added to microtiter wells coated with ^{14}C-labeled rat skin type I collagen [6,000 cpm/well (specific radioactivity 300 cpm/µg); 50 µl of medium added per well; Figure 15A] or ^{14}C-labeled type IV collagen [2,100 cpm/well (specific radioactivity 30,000 cpm/µg); 100 µl of medium added per well; Figure 15B] in the presence of increasing amounts of the recombinant human MI. Incubations were done at 37° C for 3 h (type I collagen) or 16 h (type IV collagen) in a total volume of 200 µl including 50 mM Tris-HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5 and 10 mM N-ethylmaleimide plus 2 mM PMSF. Radioactivity released to the supernatants was determined, and results are expressed as percentages of the radioactivity released in the absence of inhibitor (% inhibition).

3. E. coli - derived recombinant human MI (Table 9) and CHO cell-derived recombinant human MI (Table 10) both inhibited the degradation of type I collagen which occurs in the presence of tumor cells. The tumor cells used were c-Ha-ras transfected rat embryo fibroblasts (4R cells), since they secrete large amounts of metalloproteinases and actively degrade collagen and connective tissue (Alvarez et al., J.

TABLE 11

Effect of E. coli-derived recombinant human MI on the degradation of connective tissue matrices by 4R cells

Inhibitor concentration (μg/ml)	Matrix degradation		Inhibition (%)
	cpm/dish	% of total matrix	
0	57,590	35.9	0
0.1	56,540	33.2	1.8
1.0	42,550	25.5	26.2
10.0	25,320	14.1	56.1
25.0	19,740	11.2	65.8

[^{3}H]Proline-labeled matrices produced by rat smooth muscle cells in culture were prepared as described (Jones and De Clerck, Cancer Res., supra). They contained 15% of [^{3}H]proline in the form of glycoproteins and 85% in the form of type I and type III collagens. 4R cells were plated on the matrices at 2 x 10^5 cells/35 mm dish with 2 ml of the medium described in Table 9. Medium was changed daily with the indicated concentrations of MI also included daily with the fresh medium. Degradation of matrices was determined by measuring radioactivity released to the supernatants. The cpm/dish results represent the means of cumulative [^{3}H]proline release (above release for background cases with no cells) after 6 days for quadruplicate dishes.

Figure 16 shows the cumulative degradation on a daily basis for the experiment described in Table 11. In Figure 16, the symbols correspond to MI concentrations used, as follows: o, 0 μg/ml; o, 0.1 μg/ml; 1 μg/ml; , 10 μ/ml; , 25 μg/ml.

TABLE 12

Effect of CHO cell-derived recombinant human MI on the degradation of connective tissue matrices by 4R cells

Inhibitor concentration (μg/ml)	Matrix degradation		Inhibition (%)	Cell Number at day 6 (x10^{-6}/dish)
	cpm/dish	% of total matrix		
0	69,330	62	0	4.12 ± 0.18
0.05	64,220	57	8	4.50 ± 0.07
0.5	39,154	35	44	4.36 ± 0.21
5.0	20,314	18	71	4.56 ± 0.07
5.0 added only on days 4, 5	47,920	43	31	4.57 ± 0.07

Experimental details as for Table 11. In this experiment, it was demonstrated that the presence of inhibitor had no effect on the growth of cells, as judged by counting the number of cells present after trypsinization at day 6 (see column ti led 'cell number at day 6').

Figure 17 shows the cumulative degradation on a daily basis for the experiment described in Table 12.

solution (1%, v/v), and 10% (v/v) FBS. Subconfluent cultures were briefly trypsinized (1-2 min), collected in serum-containing medium, and suspended in PBS at a final concentration of 5 x 10^5 cells/ml. Cell viability was 97% as determined by trypan blue exclusion.

The animals used for the model were C57BL6 J mice, obtained from Jackson Laboratories (Maine), and observed for 1 week in the animal facility prior to the start of experiments. MI-treated animals (9) were injected with CHO cell-derived recombinant human MI (prepared as described in Example 10; 4.45 mg/ml in sterile PBS) into the peritoneal cavity (0.25 ml per injection = 1.1 mg per injection). Control animals were injected with 0.25 ml sterile PBS. The injections were done 13 h and 1 h prior to injection of tumor cells (7 animals) or at the time of tumor cell injection (2 animals). All animals then received additional injections of MI (treated animals) or vehicle (control animals) at 12 h intervals for a total of 5.5 days after injection of tumor cells. The B16 melanoma cells were injected into the lateral tail vein of each mouse (1.25 x 10^5 cells in 0.25 ml). All injections were alternated between MI-treated and control animals. Two weeks after injection of tumor cells, animals were sacrificed by CO_2 euthanasia and lungs were examined for the presence of surface tumor colonies after intratracheal injection of Bouin's solution. Each lung was dissected into 5 separate lobes and colonies on each lobe were counted under a dissecting microscope. Results are given in Table 13.

TABLE 13

Formation of lung nodules after injection of B16 tumor cells into MI-treated vs. control mice

Group	Number of animals	Number of lung nodules in each animal	Mean number of lung nodules (± standard error)
MI-treated	9	1,3,5,6,6,8,107,10*,80*	25.9 ± 4.1
Control	9	7,37,45,67,82,111,127,132,264	96.9 ± 7.9

* The 2 animals that were not treated with MI 13 h and 1 h prior to injection of tumor cells.

The results of Table 13 indicate a substantial and highly significant ($0.01 < p < 0.05$ by the Wilcoxon rank sum test) reduction in the appearance of lung tumor nodules as a result of the MI treatment.

EXAMPLE 13

Hematopoietic Activity of Recombinant Human Metalloproteinase Inhibitor.

Erythroid potentiating activity of recombinant human MI was demonstrated using a one-stage in vitro assay for BFU-E (burst forming units-erythroid) [Dukes et al., Experimental Hematology 13, 59-66 (1985)]. Peripheral blood was obtained from a normal volunteer donor and heparinized. Mononuclear cells were removed by centrifugation on Ficoll-Hypaque (Pharmacia) at 400 x g for 30 min. Cells were cultured at 4.1 x 10^5 cells per 35 mm dish in Iscove's modification of Dulbecco's medium, containing 0.8% (w/v) methyl cellulose, 30% (v/v) fetal calf serum, and 1.27 U/ml erythropoietin (AM-EPO, PC grade recombinant; Amgen Inc.). The recombinant human MI (derived from CHO cells; prepared as described in Example 10) was added at the indicated concentrations (Table 14) prior to plating the cells. After 10 days of incubation in a humidified atmosphere of 95% air-5% CO_2 at 37° C, colonies consisting of 3 or more subcolonies of erythroid cells or large single accumulations of erythroid cells (>300 cells) were scored as BFU-derived colonies. For each BFU-E determination, the colonies in the central 20% of the volume of 5 replicate dishes were counted. Results are given in Table 14.

biological properties of naturally-occurring metalloproteinase inhibitor, said DNA sequence selected from among:

(a) the DNA sequence set out in Figure 1 or Figure 2 or their complementary strands;

(b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and

(c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

13. A procaryotic or eucaryotic host cell transformed or transfected with a DNA sequence according to Claim 12 in a manner allowing the host cell to express said polypeptide product.

14. A polypeptide product of the expression of a DNA sequence of Claim 12 in a procaryotic or eucaryotic host.

15. A purified and isolated DNA sequence coding for procaryotic or eucaryotic host expression of a polypeptide having the primary structural conformation and biological properties of metalloproteinase inhibitor.

16. A cDNA sequence according to Claim 15.

17. A genomic DNA sequence according to Claim 15.

18. A DNA sequence according to Claim 15 wherein said DNA sequence codes for human metalloproteinase inhibitor.

19. A DNA sequence according to Claim 18 and including one or more codons preferred for expression in E. coli cells.

20. A DNA sequence according to Claim 15 having the sequence set out in Figure 2.

21. A DNA sequence according to Claim 15 and including one or more codons preferred for expression in yeast cells.

22. A DNA sequence according to Claim 15 covalently associated with a detectable label substance.

23. A DNA sequence coding for a polypeptide fragment or polypeptide analog of naturally-occurring metalloproteinase inhibitor.

24. A DNA sequence as in Claim 23 coding for methionyl metalloproteinase inhibitor.

25. A biologically functional plasmid or viral DNA vector including a DNA sequence according to Claim 12.

26. A procaryotic or eucaryotic host cell stably transformed or transfected with a DNA vector according to Claim 25.

27. A polypeptide product of the expression in a procaryotic or eucaryotic host cell of a DNA sequence according to Claim 15.

28. A synthetic polypeptide having part or all of the amino acid sequence as set forth in Figure 2 and having one or more of the in vitro biological activities of naturally-occurring metalloproteinase inhibitor.

29. A synthetic polypeptide having part or all of the secondary conformation of part or all of the amino acid sequence set forth in Figure 2 and having a biological property of naturally-occurring human metalloproteinase inhibitor.

30. A process for the production of a polypeptide having part or all of the primary structural conformation and one or more of the biological properties of naturally occurring metalloproteinase inhibitor, said process comprising:
growing, under suitable nutrient conditions, procaryotic or eucaryotic host cells transformed or transfected with a DNA vector according to Claim 25, and isolating desired polypeptide products of the expression of DNA sequences in said vector.

31. Purified and isolated human metalloproteinase inhibitor free of association with any human protein in glycosylated or nonglycosylated form.

32. A pharmaceutical composition comprising an effective amount of a polypeptide according to Claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

33. A method for inhibiting tumor cell dissemination in a mammal comprising administering an effective amount of a polypeptide according to Claim 1.

34. A method for treating rheumatoid arthritis in a mammal comprising administering an effective amount of a polypeptide according to Claim 1.

35. A DNA sequence coding for an analog of human metalloproteinase inhibitor selected from the group consisting of:

a) [Met^{-1}] metalloproteinase inhibitor; and

b) metalloproteinase inhibitor wherein one or more cysteines are replaced by alanine or serine.

36. A polypeptide product of the expression in a procaryotic or eucaryotic host cell of a DNA sequence according to Claim 35.

37. A preparation of MI which is greater than 95% pure and which comprises less than 0.5 ng of

```
att ccg gct tct atg gag cac tcg gga cca ggt ccg cgg cgc gcg cac tcg ctc

gct cgc cgc ccc cca gcc agc tct cgc ttc cgc gcc gcc agc cgc gcc ccg cgc

ctc ctc gct gca ccc cgc gac cta gag cca aga aag ttt gtg tgg cga gtg agg

gcc gga gag gag agc gcg ccc gcg gag tgc cgt cca gac cag cgc ggc ccc ggc

gga gag ggg agc gcc ccg agc cca ggc ggc ggc ggc tag ccc gag tcc gcg acc
                        -26                     -20
                        Met Gly Ala Ala Ala Arg Ser Leu Pro Leu Ala Phe
ccc gcc cct ccg ccc gcc atg ggc gcc gcc gcc cgc agc ctg ccg ctc gcg ttc
                    -10                         -1  1
Cys Leu Leu Leu Leu Gly Thr Leu Leu Pro Arg Ala Asp Ala Cys Ser Cys Ser
tgc ctc ctg ctg ctg ggg acg ctg ctc ccc cgg gcc gac gcc tgc agc tgc tcc
                      10                            20
Pro Val His Pro Gln Gln Ala Phe Cys Asn Ala Asp Ile Val Ile Arg Ala Lys
ccg gtg cac ccg caa cag gcg ttt tgc aat gca gac ata gtg atc agg gcc aaa
                              30                                    40
Ala Val Asn Lys Lys Glu Val Asp Ser Gly Asn Asp Ile Tyr Gly Asn Pro Ile
gca gtc aat aag aag gag gtg gac tct ggc aac gac atc tac ggc aac ccc atc
                                  50
Lys Arg Ile Gln Tyr Glu Ile Lys Gln Ile Lys Met Phe Lys Gly Pro Asp Gln
aag cgg att cag tat gag atc aag cag ata aag atg ttc aag gga cct gat cag
    60                                  70
Asp Ile Glu Phe Ile Tyr Thr Ala Pro Ala Ala Ala Val Cys Gly Val Ser Leu
gac ata gag ttt atc tac aca gcc ccc gcc gct gcc gtg tgt ggg gtc tcg ctg
```

FIG. 1-A

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```
att ccg gcc cgc cgt ccc cca ccc cgc cgc ccc gcc cgg cga att gcg ccc cgc

gcc cct ccc ctc gcg ccc ccg aga caa aga gga gag aaa gtt tgc gcg gcc gag

cgg ggc agg tga gga ggg tga gcc gcg cgg gag ggg ccc gcc tcg gcc ccg gct

cag ccc ccg ccc gcg ccc cca gcc cgc cgc cgc gag cag cgc ccg gac ccc cca
                                                  -26
                                                  Met Gly Ala Ala Ala Arg
gcg gcg gcc ccc gcc cgc cca gcc ccc cgg ccc gcc atg ggc gcc gcg gcc cgc
-20                                                -10
Thr Leu Arg Leu Ala Leu Gly Leu Leu Leu Leu Ala Thr Leu Leu Arg Pro Ala
acc ctg cgg ctg gcg ctc ggc ctc ctg ctg ctg gcg acg ctg ctt cgc ccg gcc
     -1   1                                       10
Asp Ala Cys Ser Cys Ser Pro Val His Pro Gln Gln Ala Phe Cys Asn Ala Asp
gac gcc tgc agc tgc tcc ccg gtg cac ccg caa cag gcg ttt tgc aat gca gat
                20                                          30
Val Val Ile Arg Ala Lys Ala Val Ser Glu Lys Glu Val Asp Ser Gly Asn Asp
gta gtg atc agg gcc aaa gcg gtc agt gag aag gaa gtg gac tct gga aac gac
                        40                                      50
Ile Tyr Gly Asn Pro Ile Lys Arg Ile Gln Tyr Glu Ile Lys Gln Ile Lys Met
att tat ggc aac cct atc aag agg atc cag tat gag atc aag cag ata aag atg
                              60                                        70
Phe Lys Gly Pro Glu Lys Asp Ile Glu Phe Ile Tyr Thr Ala Pro Ser Ser Ala
ttc aaa ggg cct gag aag gat ata gag ttt atc tac acg gcc ccc tcc tcg gca
```

FIG. 2-A

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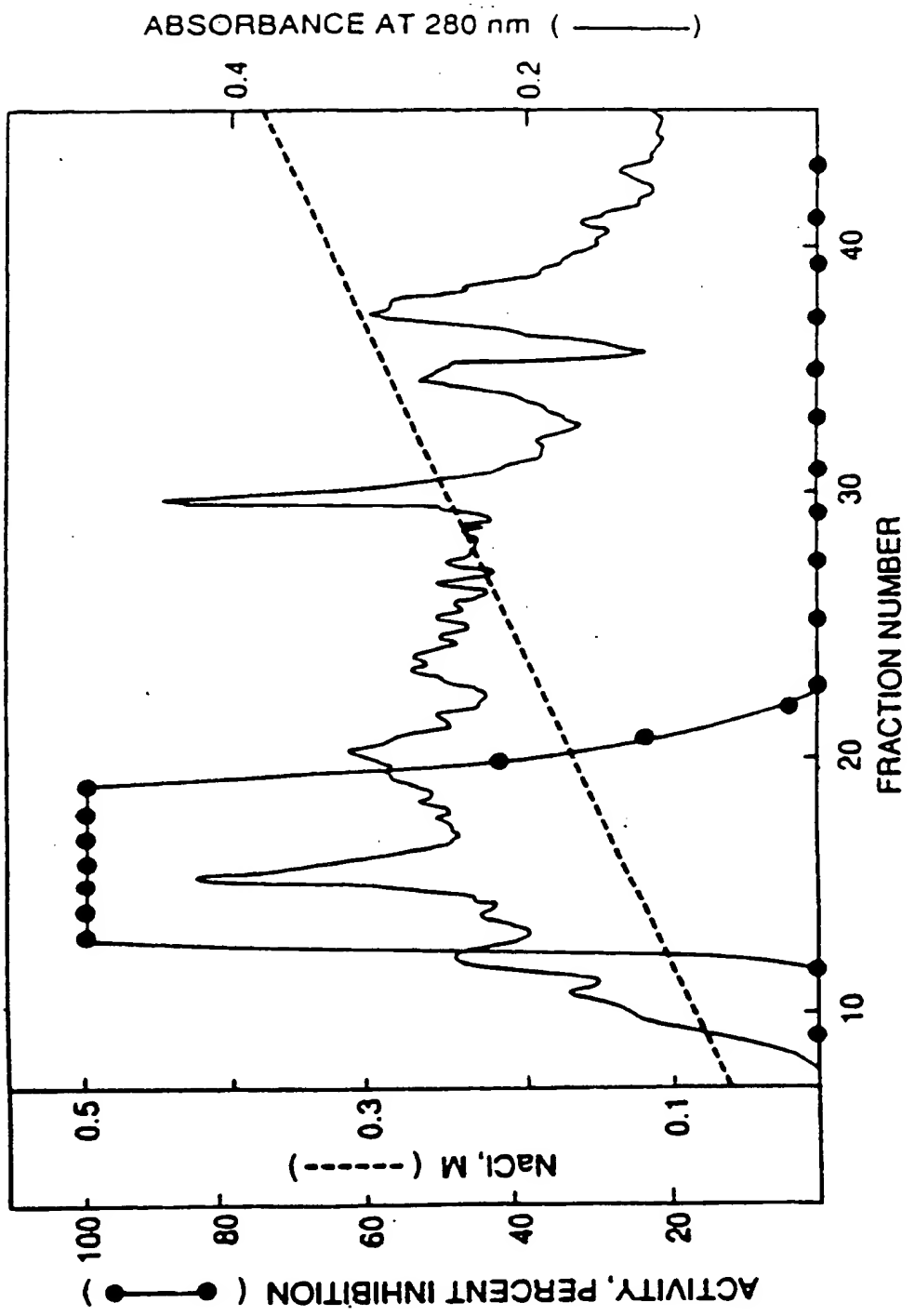
ABSORBANCE AT 280 nm (———)
0.4
0.2
10
20
30
40
FRACTION NUMBER
0.5
0.3
0.1
NaCl, M (------)
100
80
60
40
20
ACTIVITY, PERCENT INHIBITION (●——●)

FIG. 3

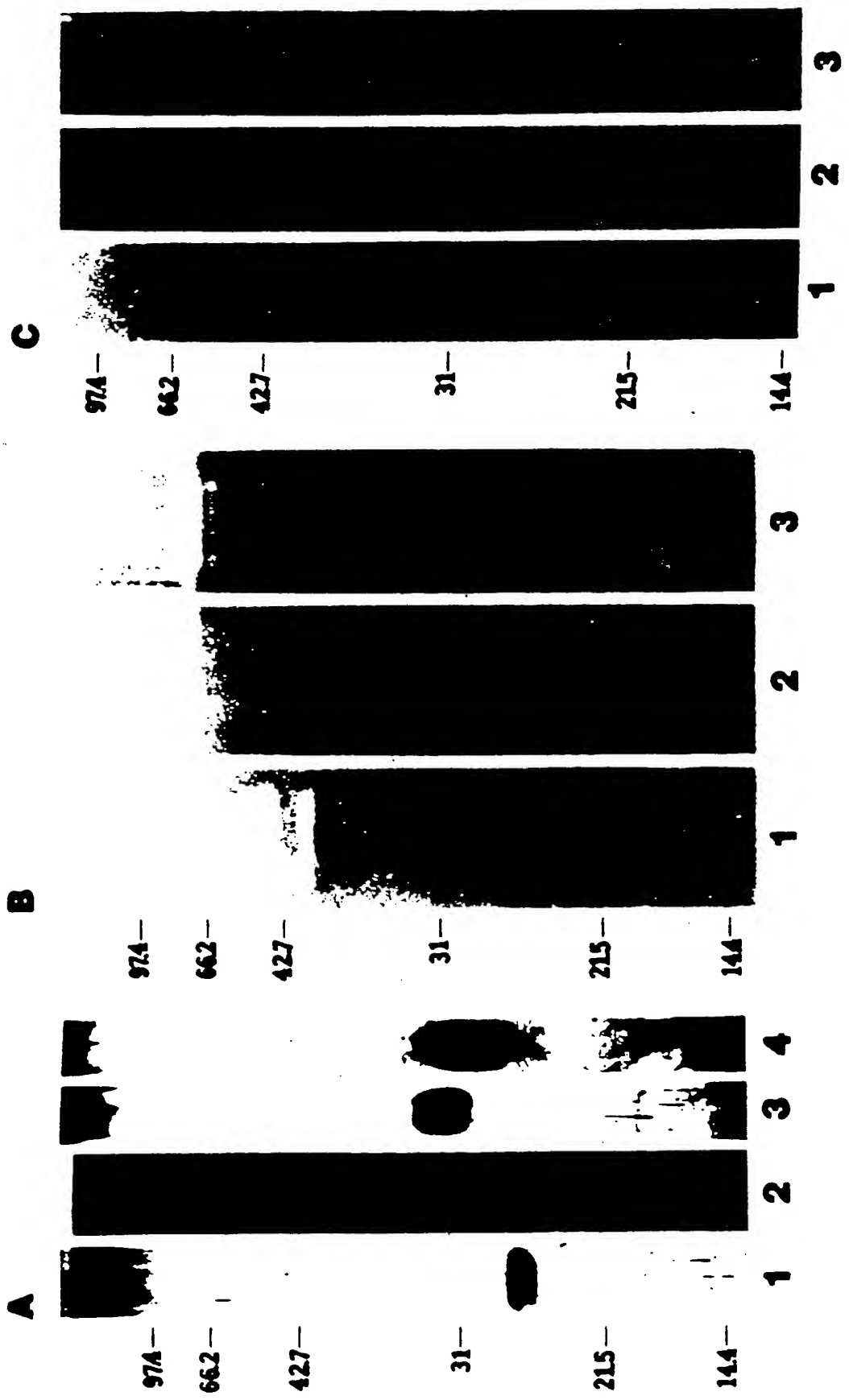

FIG. 5

FIG. 7

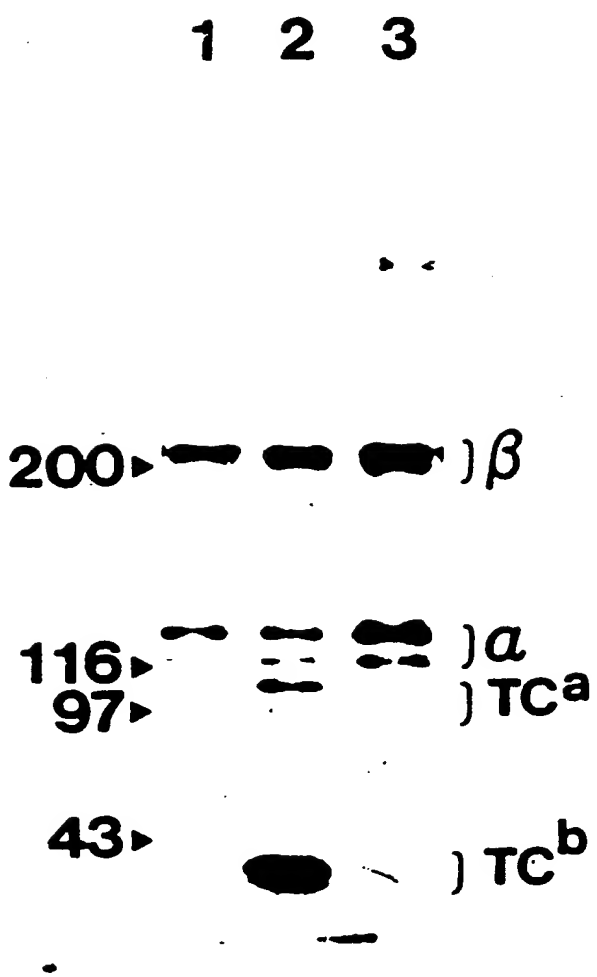

FIG. 9

```
        10          20          30          40          50          60
CTAGAAAAAA CCAAGGAGGT AATAAATAAT GTGTTCTTGT TCTCCTGTAC ACCCTCAACA
    TTTTTT GGTTCCTCCA TTATTTATTA CACAAGAACA AGAGGACATG TGGGAGTTGT

        70          80          90          100         110         120
AGCTTTTTGT AACGCTGATG TAGTTATCCG TGCAAAAGCT GTTTCTGAAA AAGAAGTTGA
TCGAAAAACA TTGCGACTAC ATCAATAGGC ACGTTTTCGA CAAAGACTTT TTCTTCAACT

        130         140         150         160
TTCTGGTAAC GACATCTACG GTAACCCGAT CAAAAG
AAGACCATTG CTGTAGATGC CATTGGGCTA GTTTTCCTAG
```

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FIG. 10-B

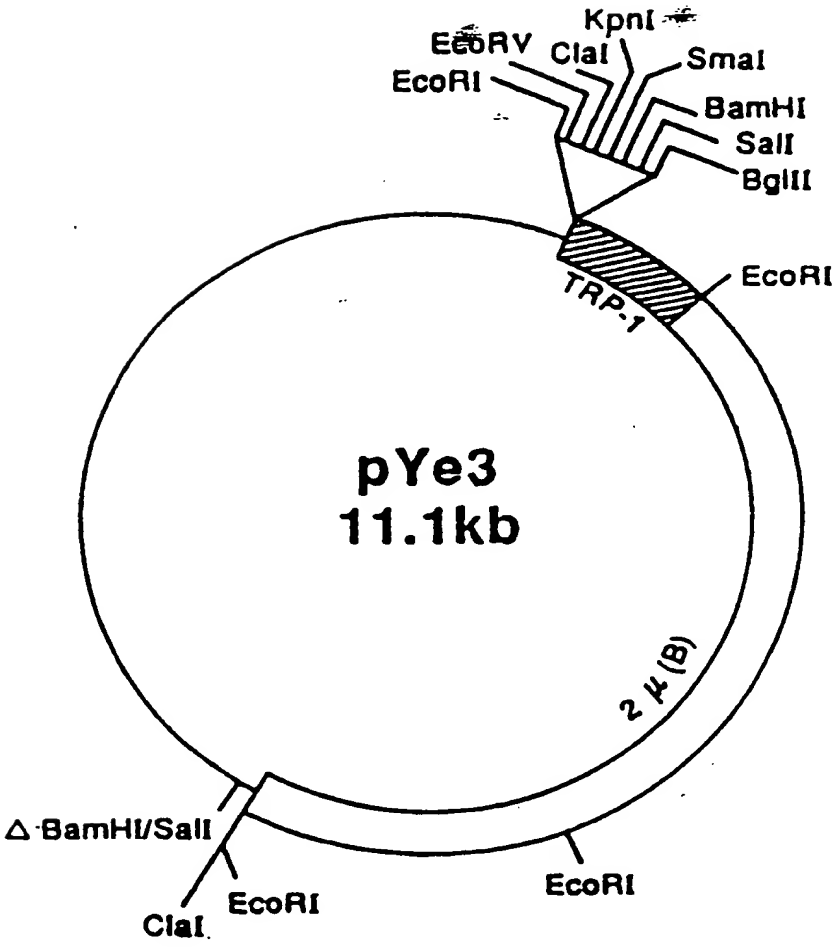

KpnI
EcoRV
ClaI
SmaI
EcoRI
BamHI
SalI
BglII
EcoRI
TRP-1
pYe3
11.1kb
2 μ (B)
Δ BamHI/SalI
EcoRI
ClaI
EcoRI

FIG. 11

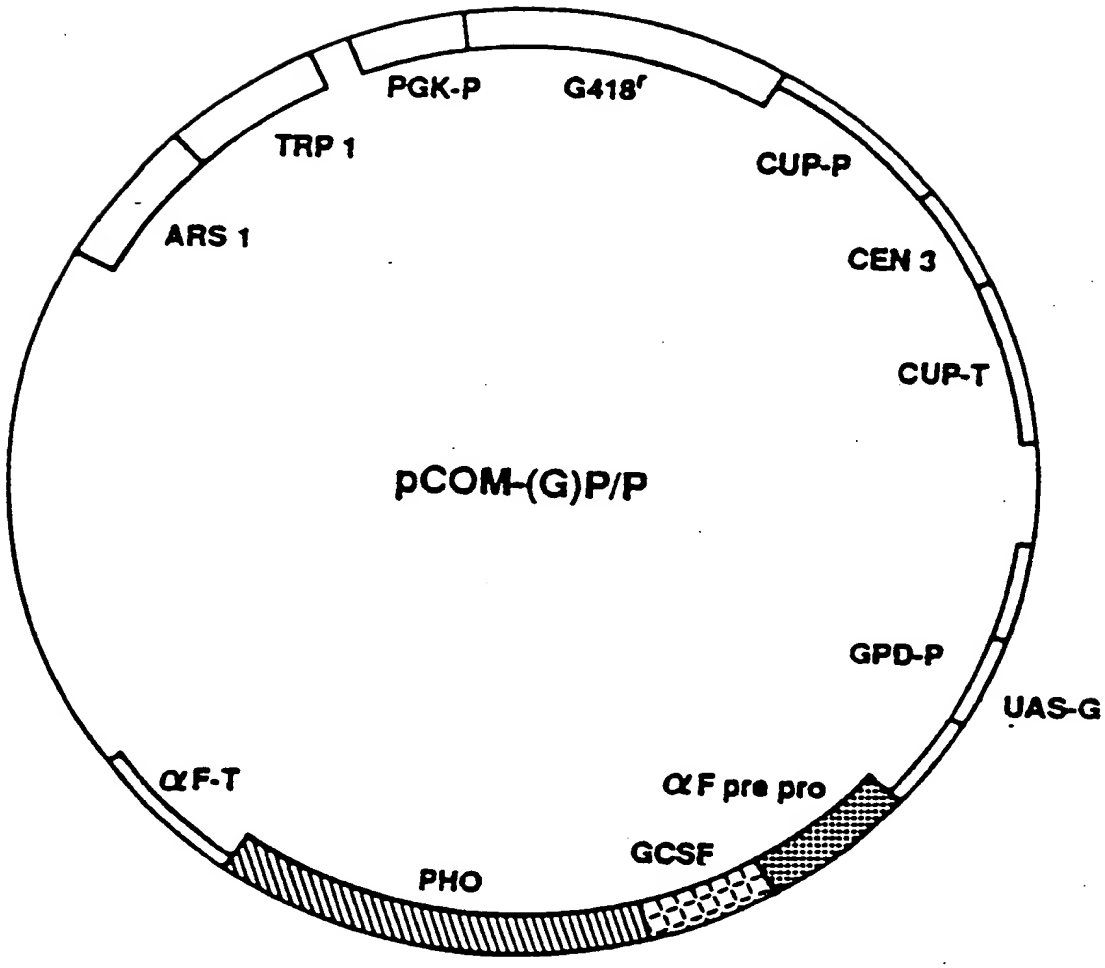

PGK-P
G418r
TRP 1
CUP-P
ARS 1
CEN 3
CUP-T
pCOM-(G)P/P
GPD-P
UAS-G
αF-T
αF pre pro
GCSF
PHO

FIG. 13

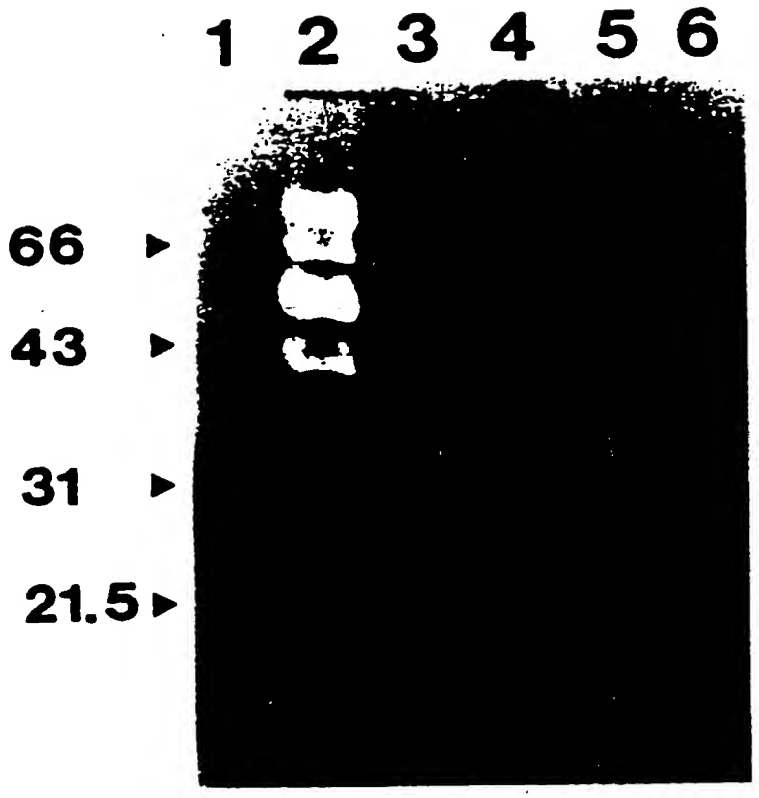


FIG. 15-A

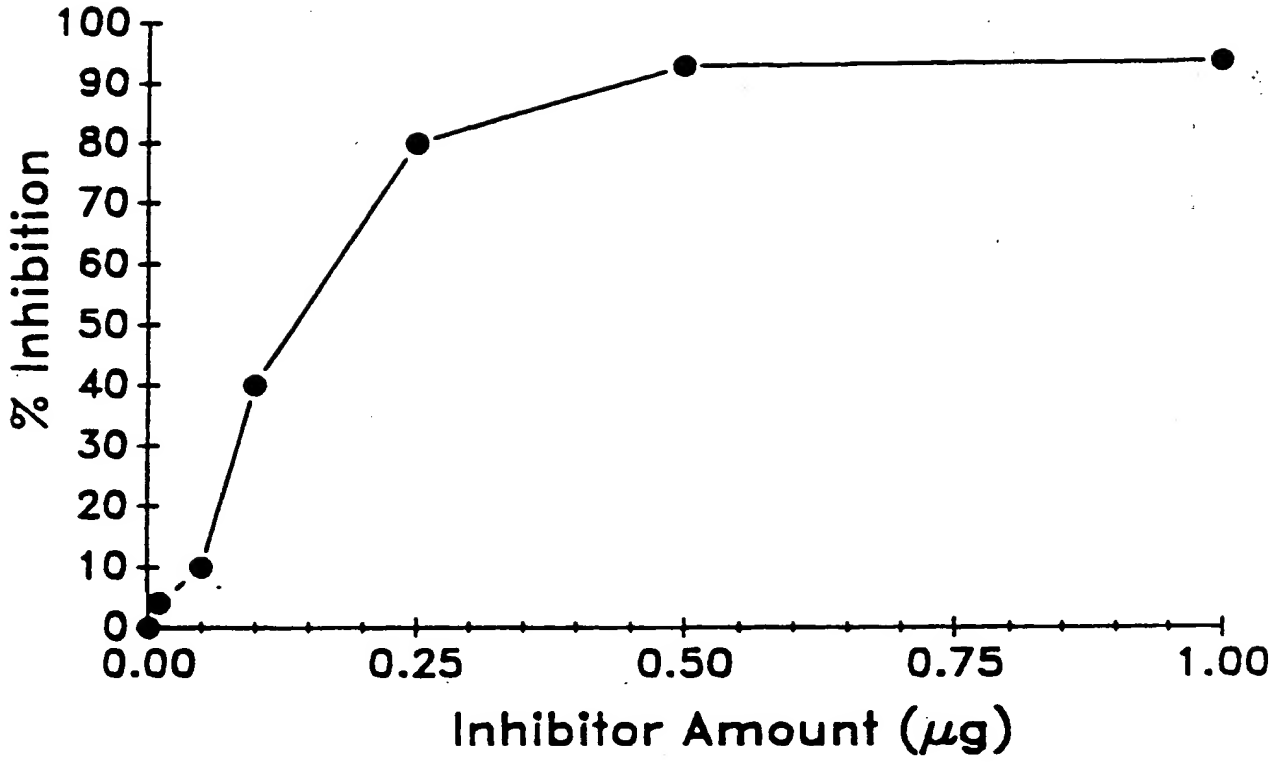

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
0.00
0.25
0.50
0.75
1.00
Inhibitor Amount (μg)

FIG. 16

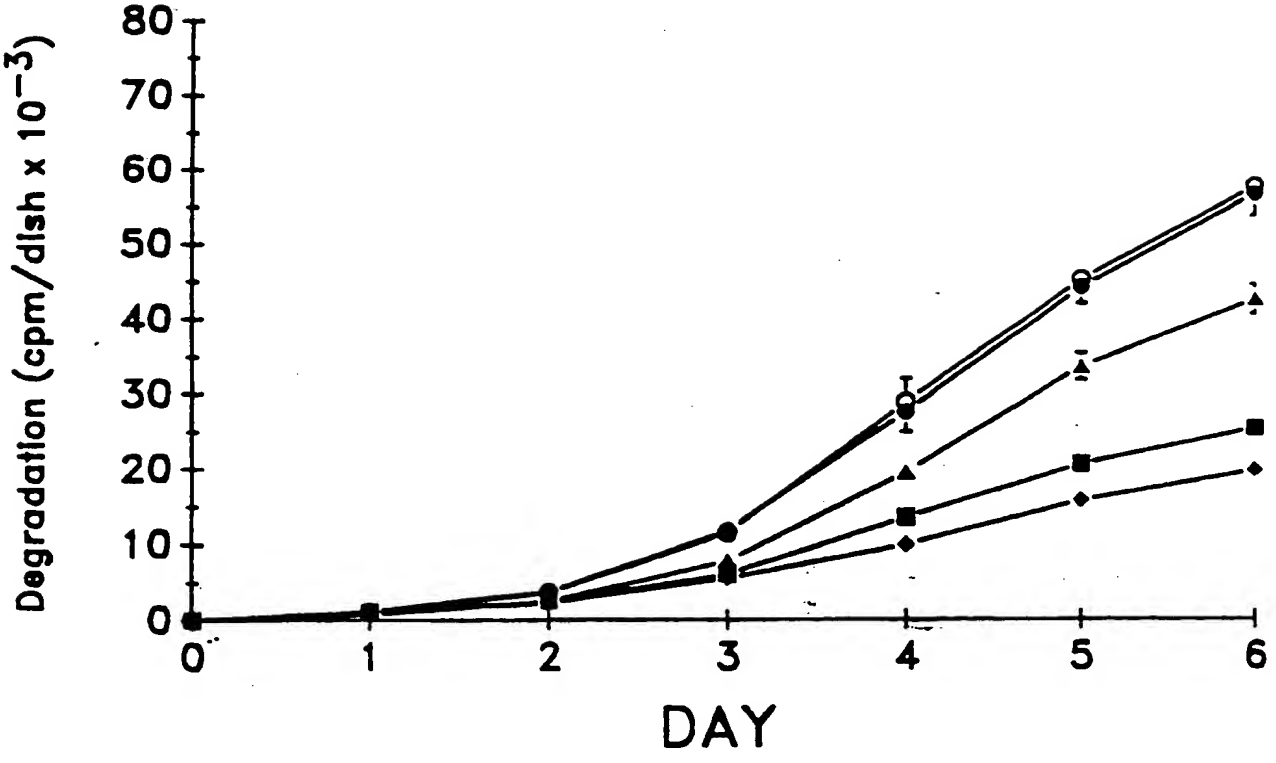


FIG. 18-A

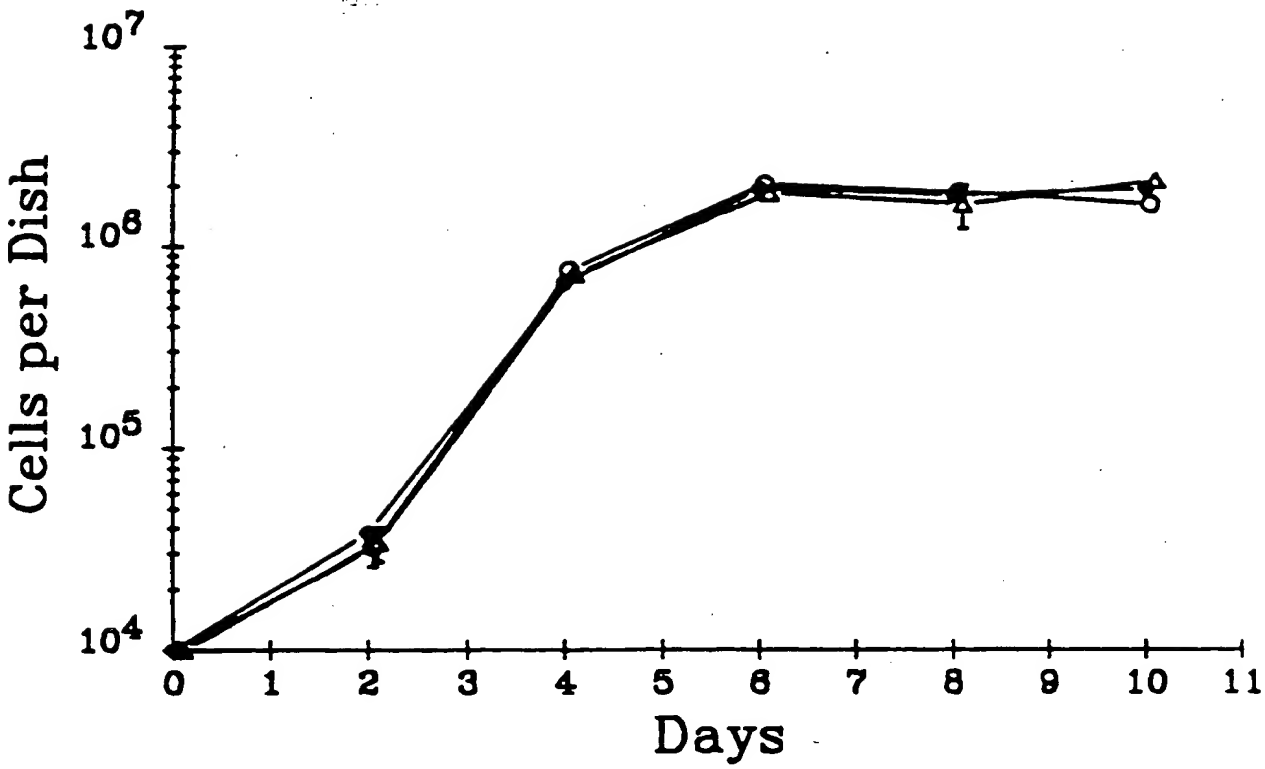
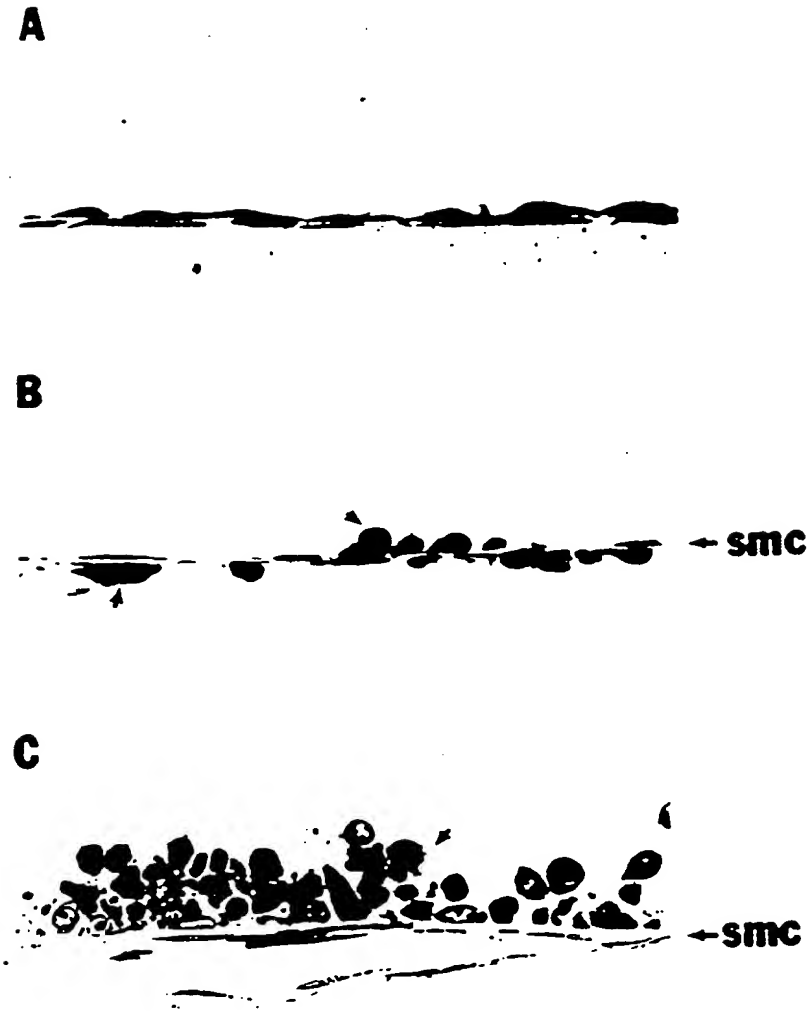


FIG. 19



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(54) Metalloproteinase inhibitor.

(57) A novel metalloproteinase inhibitor, analogs thereof, polynucleotides encoding the same, and methods of production, are disclosed. Pharmaceutical compositions and methods of treating disorders caused by excessive amounts of metalloproteinase are also disclosed.

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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D,X	EP-A-0189784 (G.D. SEARLE & CO.) * whole document *	1-4,7,9,10,15,16,18,19,21,23,27,31,32,37	
X	EP-A-0188312 (CELLTECH LTD.) * whole document *	1-4,7,9,10,15,16,18,19,21,23,27,31,32,37-39	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
X	DATABASE WPIL/DERWENT abstract no. 88-288910 (41), 1988, Derwent Publications Ltd., London, GB; & JP - A - 63210665 (FUJI YAKUHIN KOGYO) 01.09.1988 * abstract *	38,39	
P,X	PROC. NATL. ACAD. SCI. USA vol. 87, April 1990, pages 2800-2804, Washington DC, US; T.C. BOONE et al.: "cDNA cloning and expression of a metalloproteinase inhibitor related to tissue inhibitor of metalloproteinases" * whole article *	1-5,7-10,12-16,18-20,23-32,35,36	
E	WO-A-9011287 (UNITED STATES OF AMERICA et al.) * whole document *	1-4,6-32,35-39	
E	EP-A-0404750 (WASHINGTON UNIVERSITY) * whole document *	1,8-10,31	
T	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 23, 15 August 1990, pages 13933-13938, Baltimore, US; W.G. STETLER-STEVENSON et al.: "Tissue Inhibitor of Metalloproteinases-2 (TIMP-2) mRNA Expression in Tumor Cell Lines and Human Tumor Tissues" * whole article *	1-4,7-10,15,16,18,20,23,27,31,32,38	